The International Journal of Biochemistry & Cell Biology 45 (2013) 1679–1689

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Lipid raft regulates the initial spreading of melanoma A375 cells by modulating β1 integrin clustering

Ruifei Wang[a,1], Jiajia Bi[a,1], Khamal Kwesi Ampah[a], Chunmei Zhang[b], Ziyi Li[a], Yang Jiao[a], Xiaoru Wang[a], Xueqing Ba[a,*], Xianlu Zeng[a,**]

[a] Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China
[b] Department of Cell Biology, School of Norman Bethune Medical Science, Jilin University, Changchun 130021, China

ARTICLE INFO

Article history:
Received 15 December 2012
Received in revised form 28 April 2013
Accepted 29 April 2013
Available online xxx

Keywords:
Melanoma cell spreading
Lipid raft
β1 Integrin clustering
Focal adhesion
Adaptor proteins

ABSTRACT

Cell adhesion and spreading require integrins-mediated cell–extracellular matrix interaction. Integrins function through binding to extracellular matrix and subsequent clustering to initiate focal adhesion formation and actin cytoskeleton rearrangement. Lipid raft, a liquid ordered plasma membrane microdomain, has been reported to play major roles in membrane motility by regulating cell surface receptor function. Here, we identified that lipid raft integrity was required for β1 integrin-mediated initial spreading of melanoma A375 cells on fibronectin. We found that lipid raft disruption with methyl-β-cyclodextrin led to the inability of focal adhesion formation and actin cytoskeleton rearrangement by preventing β1 integrin clustering. Furthermore, we explored the possible mechanism by which lipid raft regulates β1 integrin clustering and demonstrated that intact lipid raft could recruit and modify some adaptor proteins, such as talin, α-actinin, vinculin, paxillin and FAK. Lipid raft could regulate the location of these proteins in lipid raft fractions and facilitate their binding to β1 integrin, which may be crucial for β1 integrin clustering. We also showed that lipid raft disruption impaired A375 cell migration in both transwell and wound healing models. Together, these findings provide a new insight for the relationship between lipid raft and the regulation of integrins.

1. Introduction

Cell migration plays a central role in a wide variety of biological phenomena, including embryogenesis, inflammatory response, wound healing and cancer metastasis (Lauffenburger and Horwitz, 1996). *In vivo*, studies suggest that in order to migrate to a new tissue site, various cells, such as leukocyte and cancer cell, must cross through the connective tissue barriers, which requires cell-extracellular matrix (ECM) interaction (Geho et al., 2005; Werr et al., 1998). *In vitro*, studies suggest that cell migration on two-dimensional substrate requires cell adhesion and spreading in response to ECM, which depends on the best-studied adhesion receptors – integrins (Li et al., 2010).

Integrins, composed of both α and β subunit, play a crucial role in cell-fate decision such as cell differentiation, proliferation and migration (Streuli, 2009). During cell adhesion, individual integrin recognizes and binds to unique ECM. Upon ligand binding, integrins undergo conformational changes leading to outside-in signaling and triggering the recruitment of cytoskeletal adaptor proteins (Hynes, 2002). On the other side, the signals within the cell can also propagate through integrins and provide reversible regulations over integrin-ligand binding affinity (Liu et al., 2000). During cell spreading, integrins cluster laterally within the membrane and then associate with numerous proteins to form focal adhesion. Depending on focal adhesion formation, the cell cytoskeleton is physically linked to the ECM, multiple signaling events are initiated, and cell shape is changed from a round, spheroid morphology to an irregular flattened shape (Holly et al., 2000). As spreading on the ECM, cells start the migration process. At present, although integrin clustering has been shown to play an important role in focal adhesion formation and cell spreading, the mechanisms responsible for integrin clustering remain poorly understood.

Early studies suggested that integrin affinity for ECM ligand influences its clustering (Dogic et al., 1998). More recently,

Abbreviations: ECM, extracellular matrix; MβCD, methyl-β-cyclodextrin; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MES, 4-morpholineethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CTXB, cholera toxin subunit B; siRNA, small interference RNA; EMT, epithelial–mesenchymal transition; MMPs, matrix metalloproteinases.

* Corresponding author. Tel.: +86 431 85098837.
** Corresponding author. Tel.: +86 431 85099317; fax: +86 431 85098735.
E-mail addresses: baxq755@nenu.edu.cn (X. Ba), zengx779@nenu.edu.cn (X. Zeng).
[1] The authors contributed equally to this work.

1357-2725/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biocel.2013.04.031

evidences have shown that besides ECM ligand, the recruitment of numerous cytoplasmic proteins, such as adaptors and kinases, to the integrin cytoplasmic tail is also required for integrin clustering at the cell surface (Cluzel et al., 2005; Welf et al., 2012). Interestingly, by binding to membrane lipid, some adaptors can increase their affinity for integrin and induce integrin conformational change (Cluzel et al., 2005; Legate and Fässler, 2009). Some membrane molecules, such as cholesterol and glycosphingolipids, have also been shown to be involved in the regulation of integrins (Pande, 2000). All these researches imply that membrane or membrane-proximal molecules may play an important role in controlling integrin function.

Lipid raft is a liquid ordered plasma membrane microdomain, enriched in cholesterol and sphingolipid, and is believed to be involved in forming dynamic platforms within the bilayer (Simons and Toomre, 2000). Although the existence of lipid raft has been controversial in the past few years, recent data place them at the forefront of cell biology and biomembrane research (Bodin et al., 2005). Lipid raft is in principle well suited to play major roles in regulating membrane motility by excluding or including proteins selectively (Golub et al., 2004; Simons and Toomre, 2000). The role of lipid raft in regulating the function of cell surface receptor, such as EGFR, PDGFR and IGFR, has also been proposed in various cell types (Baron et al., 2003; Huo et al., 2003). Recent evidences show that integrins may be localized to lipid raft and the disruption of lipid raft inhibits integrin function (Lee et al., 2008; Runz et al., 2008). Despite the recent significant advances, the question of whether lipid raft regulates integrin clustering and cell spreading remains unclear.

A375 cells, a highly malignant melanoma cell line, have been proved to express β1 and β3 integrins abundantly on their surfaces (Oikawa et al., 2011). Here, we investigated the role of lipid raft in adhesion and spreading of A375 cells. We show that methyl-β-cyclodextrin (MβCD) treatment, which over the years has been demonstrated to disrupt the integrity of lipid raft by effectively depleting cholesterol, prevents β1 integrin-mediated initial spreading of A375 cells on fibronectin. We found that the disruption of lipid raft inhibits β1 integrin clustering which is a prerequisite for focal adhesion formation and rearrangement of actin cytoskeleton in cell spreading process. Furthermore, lipid raft regulates β1 integrin clustering by modulating the interaction of the adaptor molecules and β1 integrin *via* concentrating and modifying the adaptor proteins. These results have extended our understanding of the relationship between lipid raft and β1 integrin clustering, and underscores the importance of lipid raft integrity in β1 integrin clustering and cancer cell migration.

2. Materials and methods

2.1. Cell culture

A375 cells were purchased from the cell bank of type culture collection of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS).

2.2. Antibodies and agents

4-Morpholineethanesulfonic acid (MES), MβCD, cholesterol oxidase (C8649), 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, M2128), HRP-conjugated cholera toxin subunit B (CTXB, C3741), mAbs to phosphotyrosine (PY20), actin (AC-40), tubulin (T4026) and vinculin (V4505) were purchased from Sigma Aldrich. Human fibronectin, mAbs to αvβ3 integrin (LM609), β1 integrin (TDM29) and active β1 integrin (HUTS-4) were obtained from Millipore. mAb to active β3 integrin (AP5) was a generous gift from Peter J. Newman (Medical College of Wisconsin, Milwaukee, USA). Nystatin (CAS 34786-70-4), mAbs to β1 integrin (JB1B) and flotillin-2 (B-6), and polyclonal antibodies to α-actinin (H-300), p-FAK (Tyr 397-R) and p-FAK (Tyr 925) were purchased from Santa Cruz Biotechnology. Polyclonal antibodies to FAK (Ab-925), paxillin (phosphor-tyr31), β3 integrin (Ab-785) and paxillin (Ab-88) were purchased from Signalway Antibody. mAb to talin (MCA 725S) was purchased from Serotech. Polyclonal antibody to paxillin (Y-118) was purchased from Cell Signaling Technology. Rhodamine-conjugated phalloidin and Alexa Fluor® 488-conjugated CTXB were obtained from Molecular Probe. TRITC or FITC-conjugated goat anti-mouse IgG Ab, FITC-conjugated anti-rabbit IgG Ab and horseradish-peroxidase-conjugated secondary Abs were obtained from Jackson ImmunoResearch Laboratories. ECL Plus Western blotting detection reagents (RPN2232) were purchased from GE Healthcare.

2.3. Flow cytometry

Cells (1×10^6) were resuspended in DMEM with or without 5 mM MβCD at 37 °C. After 30 min, cells were fixed, incubated with 2 μg of isotype IgG or specific antibodies for 60 min, washed with PBS and then stained with FITC-conjugated secondary antibody. The labeled cells were washed again and then detected by a FACScan (Beckman-Counter, USA).

2.4. Small interference RNA (siRNA)

We used siRNA approaches to knockdown endogenous β1 and β3 integrins. The sense and antisense sequences of β3 integrin siRNA oligoribonucleotides were 5′-CAAGCCUGUGUCACCAUAC-3′and 5′-GUAUGGUGACACAGGCUUG-3′ (Monferran et al., 2008), and those of β1 integrin siRNA oligoribonucleotides were 5′-GCGCAUAUCUGGAAAUUUG-3′ and 5′-CAAAUUUCCAGACAUGC-GC-3′ (Cordes et al., 2006), respectively. SiRNA duplex oligoribonucleotides were synthesized by GenePharma (China). Transfection of siRNA duplexes was performed using the Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's instructions. Experiments were performed 48 h after transfection.

2.5. Cell adhesion and spreading assays

Cell suspension (200 μl/well, 5×10^5 cells/ml) was added to glass coverslips coated with fibronectin (10 μg/ml) in 24-well flat bottom tissue culture plates. After incubation for 10 min at 37 °C, adherent cells were fixed. Five fields were imaged using phase-contrast microscope (Nikon, Japan). The number of adherent cells was counted and presented as attachment percentage (100% attachment corresponds to the attachment of untreated cells exposed to fibronectin). In cell spreading assays, A375 cells were allowed to spread for 10 min, 30 min, 60 min and 120 min, respectively. In blocking experiments, cells were preincubated with 10 mM EDTA or 10 μg/ml of blocking mAbs for 60 min at 22 °C and subjected to adhesion or spreading assay. In siRNA experiments, 48 h after transfection with β1 or β3 integrin siRNA oligoribonucleotides, the cells were collected and subjected to adhesion or spreading assay. To disrupt lipid raft, cells were preincubated with 5 mM MβCD for 30 min at 37 °C.

2.6. Immunofluorescence

Cells were incubated with or without 5 mM MβCD and allowed to spread for the indicated times prior to fixation. The fixed cells were permeabilised with 0.1% Triton X-100 for 3 min, blocked